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14. ABSTRACT

The *NF2* product, Merlin, has recently been shown to inhibit p21-activated kinases (Paks), enzymes known to activate cell cycle progression and to induce changes in the actin cytoskeleton. These findings suggest that loss of Pak function might inhibit the abnormal growth and/or movement of cells lacking Merlin.

We had proposed two aims: to test if loss of all Pak function affects signaling in $NF2^{-/-}$ mouse embryo fibroblasts and Schwann cells and to test if Paks are required for tumorigenesis in an NF2 mouse model system. In the first year of this project, we determined the expression patterns of group A Paks in murine Schwann cells; constructed and tested viral expression vectors for a Pak inhibitor (the Pak inhibitor domain); crossed NF2 with $Pak1^{-/-}$ mice to initiate tumorigenesis studies; and obtained a oncogenic NF2 mutant cDNA, subcloned it into a retroviral vector, and used the resulting virus to infect $Pak1^{-/-}$ and $Pak2^{-/-}$ MEFs in preparation for syngeneic tumorigenesis studies. With these advances, we have matched and in some cases exceeded our timetable for year one.

15. SUBJECT TERMS
NF2, GTPASE, Protein Kinase, Signal Transduction, Genetic Interactions

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Table of Contents

INTRODUCTION:

The goal of this project is to determine if group A p21-activated kinases (Paks) are important elements in signaling in neurofibromatosis type II (NF2). Our hypothesis is that inactivation of the *NF2* gene disrupts a signaling pathway emanating from the small GTPase Rac and its effector, p21-activated kinase (Pak). We propose that stimulation of the Rac/Pak signaling axis in cells lacking Merlin leads to changes in transcriptional activity and cytoskeletal dynamics, ultimately resulting in enhanced cell proliferation and motility, which are hallmarks of tumorigenesis. If this hypothesis is correct, then inhibition of Pak signaling should disable the growth advantages of cells lacking Merlin. We intend to test this theory using Pak loss-of-function cells and animals.

BODY: We set ourselves two specific tasks. These were:

Task 1. To determine if Pak function is required for mitogenic or morphogenic signaling in fibroblasts and Schwann cells lacking Merlin (Months 1-24):

a. Analyze the expression level and activity of group A Paks in fibroblasts and Schwann cells (Months 1-6).
b. Analyze effects of loss of Pak function on mitogenic and morphogenic signaling in mouse embryo fibroblasts lacking Merlin (Months 6-18).
c. Analyze effects of loss of Pak function on mitogenic and morphogenic signaling in mouse Schwann cells lacking Merlin (Months 18-24).

Task 2. To investigate the influence of Pak on the formation of tumors in transgenic mice expressing a dominant negative form of Merlin (SchΔ(39-121), by determining if crossing such mice with a) transgenic mice expressing a Pak inhibitor (PID), or b) *Pak1*$^{-/-}$ mice affects their predisposition to the tumors typically seen in NF2 (Months 6-48):

a. Generate PID transgenic cells (Months 6-12).
b. Generate and analyze PID transgenic mice (Months 12-24)
c. Mate SchΔ(39-121) mice with the PID transgenics and *Pak1*$^{-/-}$ mice and analyze crosses (Months 24-48).

Progress

We have met our goals for the first year, and have added two new approaches based on the availability of new reagents (a low molecular weight Pak inhibitor) and ideas (using *Pak1*$^{-/-}$ and *Pak2*$^{-/-}$ cells in syngeneic transplants to evaluate their role in NF2 tumorigenicity). These developments are explained in detail below.

Task 1

a) We performed an immunoblot with antibodies specific to Paks 1, 2, and 3. The results show that Pak1 is the predominant group A Pak expressed in both fibroblasts and Schwann cells but that Pak2 is also expressed at reasonably high

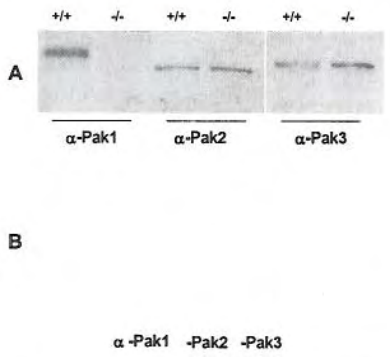

Fig. 1. Expression of Group A Paks. A) Extracts from wild-type and *Pak1*$^{-/-}$ mouse embryo fibroblasts. **B)** Extracts from Schwann cells.

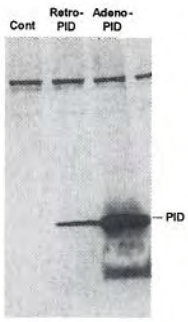

Fig. 2. Expression of Pak Inhibitor Domain (PID). Mouse embryo fibroblasts were infected with empty vector, a PID-bearing retrovirus, or a PID-bearing adenovirus. Expression was detected by anti-T7 and anti-Myc antibodies, respectively.

levels (Fig. 1). These results confirm our strategy to focus on these two isoforms of Pak in our genetic and biochemical experiments.

b) We have begun to analyze the effects of loss of Pak function on signaling in *NF2*$^{-/-}$ cells. To accomplish this task we have created both retroviral and adenoviral expression vectors encoding the Pak1 inhibitor domain (PID). When tested in mouse embryo fibroblasts, these viruses produced detectable levels of the Pak1 PID (Fig. 2). At the same time, as the result of a related research project, we have developed a small molecule inhibitor of Pak, termed A3. This compound inhibits Pak1 at a K_i of 2.4 μM in fibroblasts (Fig. 3), and is also a potent inhibitor of the other two group A Paks, Pak2 and Pak3 (not shown). A3 does not inhibit the three group B Paks at 10 μM concentrations. We are currently examining the specificity of A3 against a panel of 240 human protein kinases. A3 may thus represent an additional reagent for testing the role of group A Pak function in NF2-deficient cells. In any case, we are also going to proceed with our initial plan to test the effects of PID expression on signaling in mouse embryo fibroblasts.

c) As planned, we have not initiated studying the effects of PID expression in Schwann cells until we gather more data from the fibroblast system, because Schwann cells are relatively difficult to isolate and manipulate.

Task 2

a) We have altered the order of experiments to reflect certain logistical realities. These are that we already have available *Pak1*$^{-/-}$ and *Pak2*$^{flox/flox}$ mice, whereas we are in the process of generating transgenic mice expressing the PID. For these reasons we have focused our initial efforts on task 2c: to cross mice transgenic expressing dominant negative NF2 in Schwann cells (SchΔ(39-121))(1) with *Pak1*$^{-/-}$ mice. We have begun this experiment in earnest. By crossing, we obtained two groups of approximately 30 mice each: SchΔ(39-121) *Pak1*$^{+/+}$ and SchΔ(39-121) *Pak1*$^{-/-}$. As these mice age, we are watching them for the

development of Schwannomas and other malignancies. Depending on the outcome of this experiment, we will then engage in the initial proposed sub-aim of creating and testing the transgenic expression of the PID domain in mice, as outlined in the original proposal.

b) We have added a new task to those originally specified in the grant proposal. Given the length of time required to evaluate disease in the SchΔ(39-121) model (1 - 1.5 yr), we plan to supplement this approach with syngeneic graft tumorigenesis studies. We plan to transduce a dominant negative form of NF2 (the NF2 BBA mutant (2)) into MEFs (see Fig. 1) and/or Schwann cells derived from C57/Bl6 wild-type, *Pak1*$^{-/-}$, or *Pak2*$^{-/-}$ mice, then inject these into wild-type C57/Bl6 mice. It has been shown previously that mouse 3T3 fibroblasts transduced with NF2 BBA are tumorigenic when injected into the flanks of nude mice, resulting in palpable tumors within 1 week of injection (2). Therefore, we can test if loss of *Pak1* or *Pak2* in MEFs affects the ability of cells expressing NF2 BBA to induces tumors in this syngraft model. These experiments should be complete within twelve months, and will give us a rapid assessment about the role of Paks in NF2 tumorigenesis.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

1. Ascertained expression levels of endogenous group A Paks in MEFs and Schwann cells.
2. Constructed and tested viral expression vectors for the Pak1 PID.
3. Crossed NF2 with *Pak1*$^{-/-}$ mice to initiate tumorigenesis model.
4. Obtained NF2 BBA mutant, subcloned into retroviral vector, and used the resulting virus to infect *Pak1*$^{-/-}$ and *Pak2*$^{-/-}$ MEFs in preparation for syngeneic tumorigenesis studies.

REPORTABLE OUTCOMES:

There are not as yet any reportable outcomes.

CONCLUSION:

We have engaged in the initial steps of this project in line with our overall plans. There are two new approaches that we have added: i) the use of a small molecule Pak inhibitor to supplement our cell-based studies and ii) syngeneic graft studies as a quick readout of Pak's role in

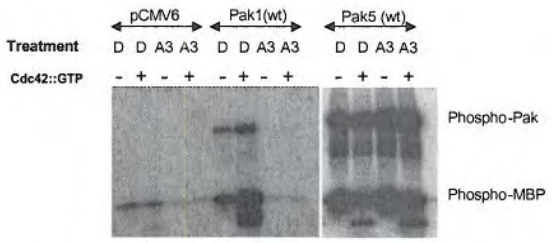

Figure 3: **Pak1 inhibitor.** Transfected Pak1 or Pak5 was immuno-precipitated from 293 cells (48 hours post transfection, last 18 hours in serum-free media) with anti-Myc antibody (9E10) washed into Phosphobuffer and treated with 30 μM A3 or equivalent amounts of DMSO (D) for 5 minutes prior to addition of myelin basic protein (MBP)/ATP, +/- Cd42 for 20 minutes

NF2-related tumorigenesis.

REFERENCES:

1. Giovannini, M., Robanus-Maandag, E., Niwa-Kawakita, M., van der Valk, M., Woodruff, J. M., Goutebroze, L., Merel, P., Berns, A., and Thomas, G., Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein., *Genes Dev., 13*, 978 (1999).
2. Johnson, K. C., Kissil, J. L., Fry, J. L., and Jacks, T., Cellular transformation by a FERM domain mutant of the Nf2 tumor suppressor gene, *Oncogene., 21*, 5990 (2002).